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Creatine Kinase Isoenzyme MB Determination on the ACA: Dependence on Serum Matrix and other Effectors

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Summary: Creatine kinase isoenzyme MB catalytic activities in human serum, determined by ACA ion exchange chromatography and immunoinhibition, differ significantly, the correlation coefficient being 0.88. The reasons for this variation are

1. interference of antibodies with the creatine kinase B subunit in the immunoinhibition assay,
2. nonreproducible elution of creatine kinase isoenzyme MB from the ion exchange resin in the ACA pack, due to varying protein concentrations in the serum samples and
3. increasing elution of creatine kinase isoenzyme MM from the ion exchange column caused by a preceding partial inactivation of creatine kinase isoenzyme MM.

Pretreatment of serum samples with a solution containing magnesium sulphate, maleate and 2-oxoglutarate (solution A) prior to determination of creatine kinase isoenzyme MB catalytic activities on the ACA significantly improves the sensitivity and specificity of the method; the correlation coefficient for the values from the ACA and immunoinhibition then becomes 0.92. Dilution of serum samples with bovine serum albumin solution is now practicable.

Bestimmung der katalytischen Aktivität von Kreatinkinase Isoenzym MB mit dem ACA: Abhängigkeit von der Serummatrix und anderen Effektoren

Zusammenfassung: Die mit der ACA-Methode (Ionenaustauschchromatographie) und der Immunoinhibitionsmethode bestimmten katalytischen Aktivitäten von Kreatinkinase Isoenzym MB in humanen Serumproben unterscheiden sich signifikant voneinander, so daß beim Vergleich der vom ACA erhaltenen Werte mit denen der Immuninhibitionsmethode 0,88 als Korrelationseffizient erhalten wird. Die Ursachen dieser Differenz sind

1. eine Interferenz der Antikörper mit der Kreatinkinase-Untereinheit B im Immunoinhibitionsansatz,
2. eine nicht reproduzierbare Elution des Kreatinkinase-Isoenzyms MB von der Ionenaustausch-Säule im ACA-Pack, verursacht vor allem durch unterschiedliche Proteinkonzentrationen in der Serumprobe und
3. eine zunehmende Kreatinkinase-Isoenzym MM-Elution von der Ionenaustausch-Säule, verursacht durch eine vorausgegangene partielle Inaktivierung dieses Isoenzyms.

Eine Vorbehandlung der Serumproben mit einer Lösung, die Magnesiumsulfat, Maleinsäure und Oxoglutarsäure enthält (Lösung A), verbessert wesentlich die Bestimmung der katalytischen Aktivitäten von Kreatinkinase Isoenzym MB mit dem ACA. Der dabei erhaltene Korrelationskoeffizient des Vergleichs der ACA-Methode mit der Immunoinhibitionsmethode beträgt 0,92. Verdünnungen der Serumprobe mit einer Rinderserum-Albuminlösung sind möglich.

[1]) This report contains essential parts of the dissertation of *C. Kaul-Kunz*.

Introduction

The myocardium is the only tissue containing relatively large amounts of creatine kinase (EC 2.7.3.2) isoenzyme MB (CK-MB). Diagnosis of myocardial injury therefore is generally possible from the appearance of this isoenzyme in the plasma together with the corresponding clinical symptoms (1). The use of creatine kinase isoenzyme MB activity in the diagnosis of myocardial infarction is thus well established (2).

Numerous methods are available for measuring the isoenzymes of creatine kinase (3, 4), each method having a different sensitivity and specificity. These include electrophoretic separation (5), ion exchange column chromatography (6), the immunoinhibition assay (7) and the radioimmunoassay (8). While electrophoresis and ion exchange column chromatography give complete separation of the three isoenzymes they are cumbersome and only adequate for detecting creatine kinase isoenzyme MB catalytic activities at peak values. A variation of ion exchange column chromatography is the method used by the ACA (Du Pont), in which a mixed anion-cation exchange chromatography column is used with a pH near the isoelectric point of creatine kinase isoenzyme MB so that most of the enzyme is not retained by the ion exchange resin (9). Its activity is then determined automatically in the eluate. Electrophoresis and radioimmunoassay are not practical for general laboratory use, but the immunoinhibition method and the ACA method are in common use.

However, a reevaluation of the ACA method was necessary because the correlation between the creatine kinase isoenzyme MB catalytic activities obtained by the ACA and immunoinhibition methods is only partial (10).

Materials and Methods

The creatine kinase isoenzyme MB from human myocardium was purified according to l.c. (11) with the modification that all steps were carried out in the presence of 1 mmol/l EGTA. The purified creatine kinase isoenzyme MB preparation was homogeneous as judged by disc gel electrophoresis (12) and contained no detectable creatine kinase isoenzyme MM (CK-MM) catalytic activity as shown by electrophoresis on cellulose acetate strips (5). Human creatine kinase isoenzyme BB was obtained by homogenization of 10 g uterus muscle in 100 ml of 0.05 mol/l Tris-HCl buffer pH 7.4 and 10000 *g* centrifugation. The supernatant contained a pure creatine kinase isoenzyme BB preparation as judged by electrophoresis on cellulose acetate strips (5).

Electrophoresis was carried out according to l.c. (5) with several modifications: 0.05 mol/l barbituric acid buffer pH 8.6, time of electrophoresis 30 min at 25 °C, development of creatine kinase bands in the cellulose acetate strips at 37 °C for 30 min on 2 g agar in 100 ml H_2O, mixed with CK-monotest at a ratio of 1+1 (vol/vol).

Quantitative determination of reduced $NADP^+$ in the creatine kinase isoenzyme bands was carried out fluorometrically, using a Zeiss PMQ-II spectrophotometer (Fa. Zeiss, Oberkochen, Germany) (excitation at 340 nm, reflected emission at 450 nm).

Total creatine kinase catalytic activity was measured on the ACA II (starting point = 0, scale factor = 0.6228). Creatine kinase MB catalytic activities were determined on the ACA II (starting point = 0, scale factor = 0.6068 or 0.8422 or 0.3096) and with the immunoinhibition method (10 min preincubation, filter 365 nm, photometer Eppendorf, 25 °C). Solution A contained 1.5 mol/l magnesium sulphate, 25 mmol/l maleic acid and 1.25 mmol/l oxoglutaric acid. The sample was mixed with solution A in a ratio of 1+9 prior to measurement of creatine kinase isoenzyme MB catalytic activities on the ACA.

The serum samples used in this assay were obtained from different persons from the intensive care unit without special selection. The lowest and highest total creatine kinase catalytic activity concentration measured was 98 U/l and 4810 U/l. Diagnosis of acute myocardial infarction was by electrocardiogram with the exception of one case, which was diagnosed from serum enzyme catalytic activities.

Isoelectric focussing of purified creatine kinase isoenzymes MM and MB was carried out with a LKB 110 ml column (Ampholine pH 3–7) according to the LKB instruction manual.

Effectors were added to serum or to bovine serum albumin containing samples in a volume ratio of 1+9.

Creatine kinase catalytic activities at 37 °C were converted to activities at 25 °C by multiplying by 0.42 (13).

The coefficients of variation (N = 25) for the methods were as follows: immunoinhibition method creatine kinase MB 8.8% (35 U/l), electrophoresis creatine kinase MB 11.8% (22 U/l), ACA creatine kinase MB 6.0% (50 U/l), ACA creatine kinase MB with sample pretreatment by solution A 5.4% (56 U/l), ACA creatine kinase 5.7% (70 U/l).

Monotests for determination of creatine kinase isoenzyme MB catalytic activities with the immunoinhibition method were obtained from Fa. Boehringer, D-6800 Mannheim and Fa. Merck, D-6100 Darmstadt, Germany. Aspartate aminotransferase (EC 2.6.1.1), alanine aminotransferase (EC 2.6.1.2) and adenylate kinase (EC 2.7.4.3) from porcine heart, glutamate dehydrogenase (EC 1.4.1.3) from bovine liver, human immunoglobulin G, organic and inorganic reagents and substrates were commercial preparations from Fa. Boehringer, Fa. Merck, Fa. Sigma, D-8000 München and Fa. Serva, D-6200 Heidelberg.

The comparision of the analytical procedures was carried out using the standardized principal component analysis.

Results

Effects of matrix composition on the creatine kinase isoenzyme MB determination by the ACA method

Constituents tested

Several constituents normally present in plasma were tested for their effect on the ACA method, using concentrations exceeding the normal range. H^+-concentration (pH 7.6, pH 7.2) lactic acid (6.0 mmol/l), cholic acid (400 mg/l), cholesterol (2 g/l), bilirubin (10 mg/l), protoporphyrin (50 µg/l), glutamate dehydrogenase (4000 U/l), aspartate amino-

transferase (4000 U/l), alanine aminotransferase (4000 U/l) and adenylate kinase (4000 U/l) did not modify the ACA creatine kinase MB values when added to purified creatine kinase MB in bovine serum albumin solutions (80 g/l).

A human serum pool containing varying contents of added creatine kinase isoenzymes BB or MM and containing no creatine kinase MB catalytic activity was tested by the ACA creatine kinase MB method (tab. 1). Creatine kinase MM is retained (99%) by the ACA column, creatine kinase BB is partially (19–40%) eluted so that falsely high creatine kinase MB catalytic activities are obtained.

Tab. 1. Effects of human creatine kinase MM and BB on the creatine kinase MB assay on the ACA. The enzymes were added in the indicated catalytic activity concentration (25 °C) to a human serum pool containing no creatine kinase activity. The creatine kinase MB activity was determined on the ACA with the factor 0.6068 and was then corrected to 25 °C by the factor 0.5829.

	Catalytic concentration added	Catalytic concentration measured by ACA (U/l)	
	(U/l, 25 °C)	Control	After dilution by solution A
Creatine kinase MM	1424	8	9
Creatine kinase BB	98	35	29
	77	25	23
	57	15	15
	38	10	9
	18	3	4

The only other known constituent which significantly influences the creatine kinase MB values obtained by the ACA is total protein. It modifies the retention of creatine kinase MB on the ion exchange column in the ACA pack (fig. 1). Increasing plasma protein or bovine serum albumin or human immunoglobulin G concentrations cause an appropriate increase of creatine kinase elution from the column as indicated by increased catalytic concentrations measured by the ACA (an increase of 1 g/l serum protein corresponds to an increase of 1 U/l, ACA factor 0.6068). Other unknown constituents present in human serum modify the elution of creatine kinase from the ACA column. Therefore, a dilution of serum is not possible, even if a solution of bovine serum albumin is used for dilution (fig. 2).

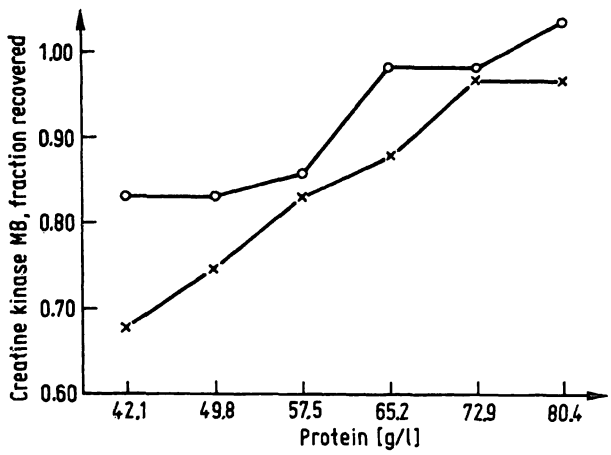

Fig. 1. Dependence of ACA creatine kinase determination on the protein concentration in the serum sample. Purified creatine kinase MB (49 U/l, determined in the ACA total creatine kinase channel, 25 °C) was added to human serum samples. x—x control, O—O sample pretreatment by solution A. Catalytic concentration was measured with the ACA factor 0.6068 and was converted to 25 °C by the factor 0.5829.

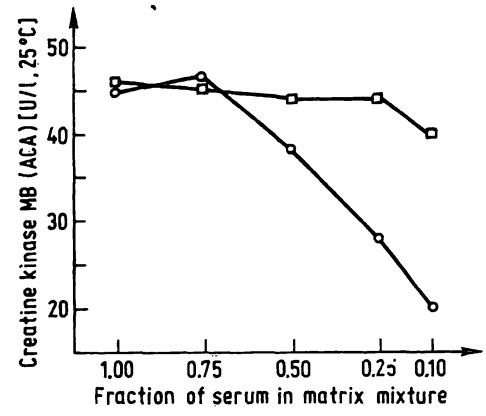

Fig. 2. Dependence of creatine kinase determination by the ACA on the dilution of serum samples. Purified creatine kinase MB (49 U/l, 25 °C) was added to a human serum sample, which was diluted with a bovine serum albumin solution (80 g/l) as indicated. O—O control, □—□ with sample pretreatment by solution A. The catalytic concentration was measured with the ACA factor 0.6068 and was then converted to 25 °C by the factor 0.5829.

Influence of solution A on creatine kinase MB determination by the ACA method

When commercially available porcine heart aspartate aminotransferase was tested for its effect on the determination by the ACA it was observed that the obtained values were in good agreement with the creatine kinase activities added to the matrix. This improved elution from the ACA column, however, was not caused by the aspartate aminotransferase but by the stabilization mixture present in the aspartate aminotransferase solution. This mixture consist-

ed of ammonium sulphate, maleate and 2-oxoglutarate. After exchanging ammonium sulphate for magnesium sulphate this solution A was tested for its influence on the determination of creatine kinase activities in human serum by the ACA (figs. 1–3, tab. 1).

Addition of solution A to serum samples reduces the effects of serum protein (fig. 1) and other serum constituents (fig. 2) on creatine kinase MB determination by the ACA. Dilution of serum samples with bovine serum albumin solutions, containing 80 g/l protein, is now possible when the diluted sample is pretreated with solution A. No effect of solution A on the elution of creatine kinase MM or MB is observed (tab. 1).

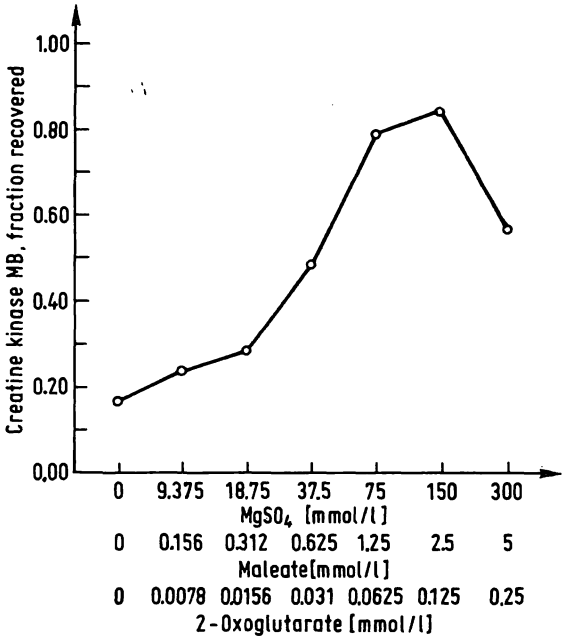

Fig. 3. Effects of pretreatment of samples with different concentrations of substances contained in solution A. Purified creatine kinase MB (49 U/l, 25 °C) was added to a bovine serum albumin solution (80 g/l). The catalytic concentration was measured on the ACA with the factor 0.6068 and was then converted to 25 °C by the factor 0.5829.

When serum samples containing added purified creatine kinase MB and creatine kinase MM are incubated at room temperature for several days, an apparent increasing creatine kinase MB catalytic activity is measured by the ACA (tab. 2). This is due to the creatine kinase MM present in the serum matrix or due to the added creatine kinase, which is not completely retained by the ion exchange column. After sample pretreatment with solution A this increased elution of creatine kinase MM from the column in the ACA pack, is not observed.

Tab. 2. Effects of inactivation of creatine kinase MM on the determination of creatine kinase MB in human serum. 140 U/l (25 °C) creatine kinase MM was added to a human serum pool. After the indicated incubation time at 25 °C total creatine kinase and creatine kinase MB catalytic activities were measured on the ACA (factor 0.3096) with and without sample pretreatment by solution A. Above values were calculated by subtracting the measured activities of the native serum pool from the activity values obtained after addition of creatine kinase MM.

		Days of incubation 0	2	7
Total creatine kinase	(U/l)	139	140	123
Creatine kinase MB	(U/l)	4	6	10
Total creatine kinase	(U/l)	140	140	115
Creatine kinase plus solution A pretreatment	(U/l)	4	4	4

Correlation of the immunoinhibition and the ACA method

The correlations of creatine kinase MB catalytic activities in the serum of 28 patients suffering from acute myocardial infarction measured by the immunoinhibition method (25 °C) and the ACA methods (25 °C) are shown in figures 4–7b and table 2. The values obtained by the ACA method (37 °C) with the original factor 0.6068 are too low for a temperature of 37 °C. Later, Du Pont corrected this factor from 0.6068 to 0.8422. When the creatine kinase MB catalytic activities are converted from factor 0.6068 to 0.8422 and are multiplied by 0.42 (13) (factor 0.5829) then the activities for a temperature of 25 °C are obtained. A comparison of these values with the activities obtained by the immunoinhibition method shows good agreement in slope (1.0), but the correlation coefficient is only 0.88 (fig. 4). Addition of solution A to the serum samples increases the slope (immunoinhibition method versus ACA method at 25 °C) to 1.29 and the correlation is improved to 0.92 (fig. 5).

When human serum samples containing varying catalytic activities of added purified creatine kinase MB are measured on the ACA in the total creatine kinase channel (25 °C) and in the creatine kinase MB channel (factor 0.3096) after pretreatment with solution A, values for total creatine kinase and creatine kinase MB are identical. Following the correction of creatine kinase MB activities obtained for the 28 serum samples after pretreatment with solution A, using the factor 0.3096 instead of 0.6068, the values compare well with the activities obtained by the immunoinhibition (fig. 6). The slope is now 1.03, the coefficient of correlation 0.92.

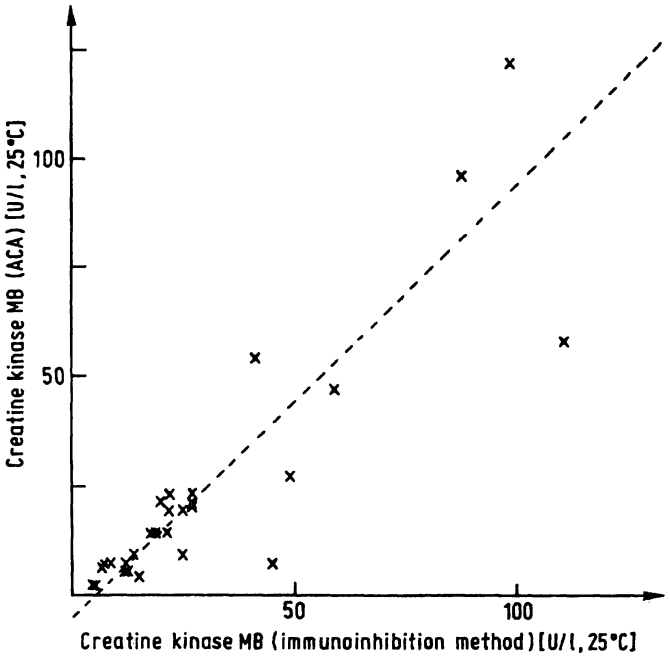

Fig. 4. Samples: human serum from persons suffering from an acute myocardial infarction. Correlation of creatine kinase MB values obtained by the immunoinhibition method (25 °C) and the ACA method as described in fig. 3.

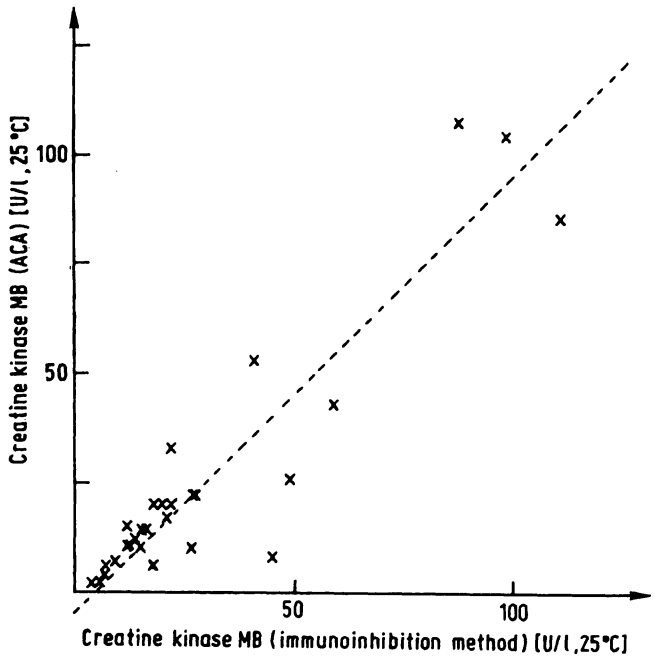

Fig. 6. Correlation as in fig. 4. Samples were pretreated with solution A prior to determination of catalytic concentration on the ACA (factor 0.3096).

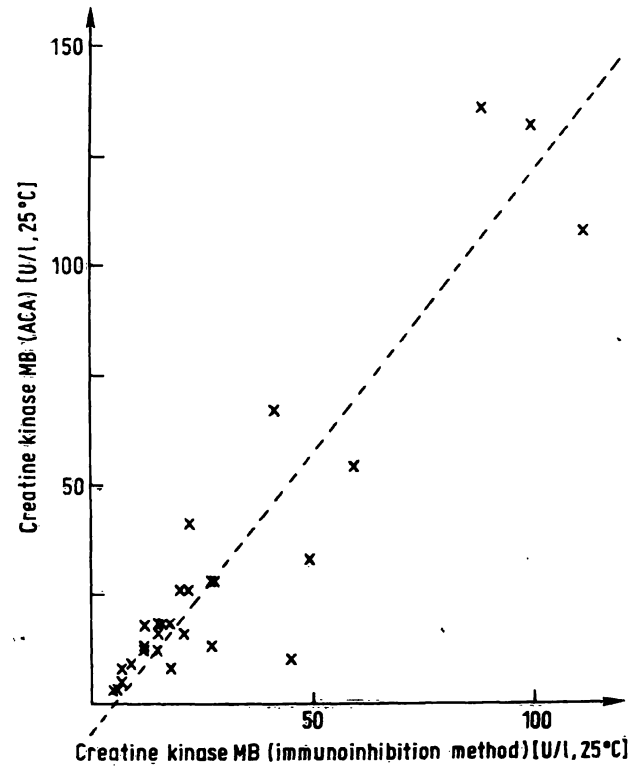

Fig. 5. Correlation as in fig. 4. The samples were pretreated with solution A prior to determination of catalytic concentration on the ACA.

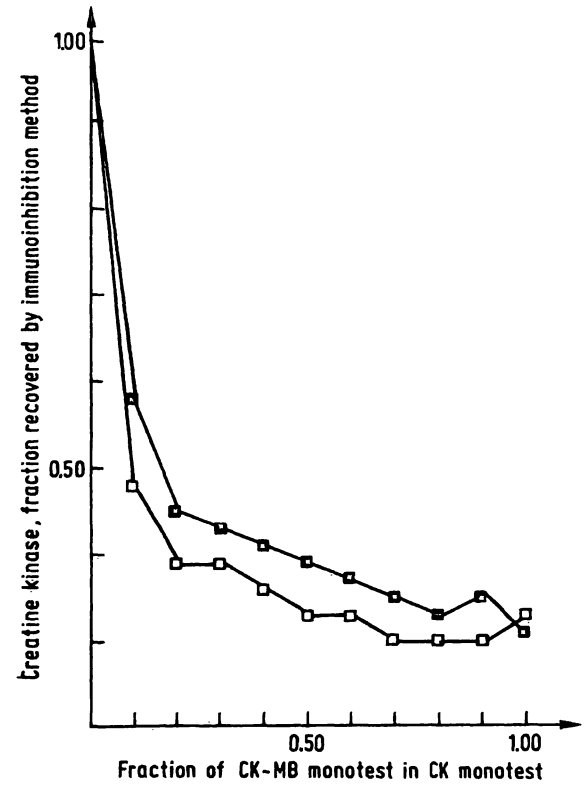

Fig. 7. Residual creatine kinase activity of a creatine kinase MB preparation added to bovine serum albumin (80 g/l) (■—■) and to a human serum pool (□—□) in dependence of the anti-creatine kinase MM antibody concentration, as determined by the immunoinhibition method. The varying antibody concentration was obtained by mixing creatine kinase and creatine kinase MB monotests as indicated.

Inhibition of creatine kinase B subunit by anti-creatine kinase MM antibodies

During the purification procedure of human heart creatine kinase MB it was observed that the creatine kinase catalytic MB activities obtained by the immunoinhibition method were always lower than the values measured by the total creatine kinase method, even when the creatine kinase MB preparations contained no detectable isoenzyme MM activity. This discrepancy was caused by a partial inhibition of creatine kinase B subunit by the anti-creatine kinase MM antibodies present in the immunoinhibition test (fig. 7). This inhibition is also observed when preparations are preincubated in 200 mmol/l N-acetylcysteine and when creatine kinase MB preparations were used which had been separated from creatine kinase isoenzyme MM by a single purification step (human heart homogenate was applied to DEAE-Sepharose A 50 and creatine kinase MB was eluted according to l.c. (11)).

Discussion

The original ACA method for determination of creatine kinase MB catalytic activities shows several limitations. Dilution of the samples is not possible and 30–40% of the creatine kinase MB in the serum sample is retained on the column (9) so that a correction of the obtained values is necessary.

When using the immunoinhibition method for measurement of creatine kinase MB activities it must be presupposed that the creatine kinase B subunit remains active after incubation of the serum with the creatine kinase MM antibodies. This cannot be guaranteed as is seen in figure 7, where an apparent 20% inhibition of the purified creatine kinase B subunit by creatine kinase MM antibodies is observed. The same inhibition by creatine kinase MM antibodies is observed when creatine kinase MB preparations are used which have been separated from the creatine kinase MM by a single chromatography step, thus largely preventing possible denaturation of the creatine kinase B molecule. Use of monotests for the immunoinhibition method from another commercial source yielded identical results. Similar observations have been reported by other authors (14). It is not clear whether the antibodies against the creatine kinase M subunit present in the immunoinhibition monotest inhibit the creatine kinase B subunit in native serum as well.

If an inhibition of creatine kinase B subunit is excluded, then the above described effects must be explained by different turnover numbers of the subunits M and B, and may be based on a differential course of inactivation of the subunits. It is known that the thermolability of the creatine kinase isoenzymes increases in the order creatine kinase isoenzyme MM, MB and BB (15). This preferential inactivation of the creatine kinase B subunits also occurs in vivo (16) and results in a shift of remaining catalytic activity toward creatine kinase MM. In either case, use of the immunoinhibition method for determination of creatine kinase MB catalytic activities is limited to serum samples where the creatine kinase subunit B activity is not affected, i.e. to serum samples from persons suffering from a fresh acute myocardial infarction. It is known (21) that the concentration of creatine kinase B remains elevated longer than its catalytic activity, and that the elimination kinetics for catalytic activity and concentration are different.

In addition, an increased creatine kinase B value from the immunoinhibition method might be difficult to interpret, if creatine kinase BB is also possibly present in the plasma. While most diseases leading to activities in plasma do not present a difficult diagnostic problem in relation to acute myocardial infarction (17), this is not true in the case of anoxic brain injury after cardiac arrest (18). If creatine kinase BB is present in serum samples which are tested for creatine kinase MB activity, the values derived from the immunoinhibition method are falsely increased by 200% of the residual creatine kinase BB activity, whereas the creatine kinase MB values obtained by the ACA are only increased by 22–29% of the residual creatine kinase BB activity.

While the ACA method is not disturbed by many of the metabolites present in plasma (tab. 1), the total protein content in the sample is decisive in obtaining acceptable values. Differences in plasma protein concentration is one of the reasons for a variable retention of creatine kinase MB on the ion exchange column. This was first shown by l.c. (19) and later in greater detail by l.c. (20).

When solution A is added to the samples, the determination of creatine kinase catalytic activity on the ACA is significantly improved and reproducible values are obtained. The effect of protein concentration in the serum sample is eliminated and dilution of serum samples with a bovine serum albumin solution is possible. Of all the methods compared, the best diagnostic sensitivity and specificity are obtained with the modified ACA method (fig. 6, tab. 3) and a significant separation is observed of the infarction samples (fig. 8). There are two reasons for the improvement of the ACA creatine kinase MB determination due to pretreatment with solution A. Since

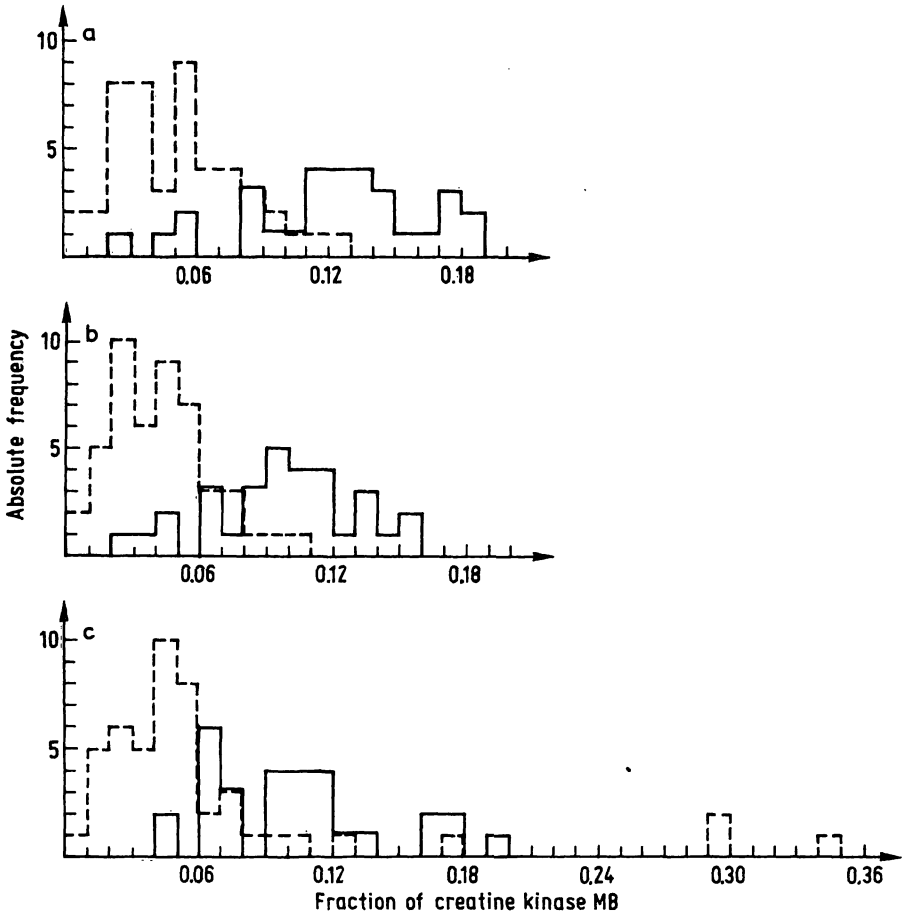

Fig. 8. Histogram of frequency of creatine kinase MB catalytic activity expressed as fraction of total creatine kinase activity. Samples from patients without (– – –) and with (——) acute myocardial infarction. N = 79. The diagnostic decision limit for acute myocardial infarction is a creatine kinase activity fraction of 0.06.
a. Determination of catalytic concentration on the ACA (factor 0.6068, corrected to 25 °C by factor 0.5829). Sensitivity 0.87, specificity 0.667, predictive value positive test – p.v. (+) – 0.627, predictive value negative test – p.v. (–) – 0.89.
b. Determination of catalytic concentration on the ACA (factor 0.6068, corrected to 25 °C by factor 0.5102) with sample pretreatment by solution A. Sensitivity 0.87, specificity 0.812, p.v. (+) 0.743, p.v. (–) 0.901.
c. Determination of catalytic concentration by the immunoinhibition method (25 °C). Sensitivity 0.935, specificity 0.729, p.v. (+) 0.69, p.v. (–) 0.946.

addition of N-acetylcysteine to the serum samples has an effect similar to the addition of solution A, an activation of creatine kinase MB with an improved elution from the column might be occurring. Secondly, additional sample pretreatment causes an increased retention of creatine kinase MM on the column in the ACA pack as seen in table 2. After incubation of purified creatine kinase MM/MB combinations in serum matrix for several days at room temperature, increasing amounts of creatine kinase MM are eluted from the column, so that apparently higher creatine kinase MB activities are measured. The ACA ion exchange chromatography is carried out at pH 6.1 and the isoelectric points of human creatine kinase isoenzymes MM and MB are 6.95 and 4.85, respectively. The partial inactivation of creatine kinase MM causes a shift of isoelectric point toward pH 6.1 as judged by a slightly faster migration of this partially inactivated creatine kinase MM in electrophoresis. As a result this modified creatine kinase MM is not retained as completely by the column as the fully active creatine kinase MM. We must assume that similar effects occur in vivo (16), so that sometimes falsely high creatine kinase MB activities are measured by the original ACA method.

Tab. 3. Comparison of analytical methods (y = bx + a). IIM = immunoinhibition method.

Compared methods	N	x	y	Sxy	r	a	b
IIM (25 °C)/ACA-MB factor 0.6068, corrected by factor 0.5829	28	29	24	694	0.88	−5.97	1.0
IIM (25 °C)/ACA-MB + sol. A, factor 0.6068, corrected by factor 0.5829	28	29	31	899	0.92	−6.68	1.29
IIM (25 °C)/ACA-MB + sol. A, factor 0.3096	28	29	25	725	0.92	−5.31	1.03

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